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Change in the content of titin and nebulin and their phosphorylation level in the quadriceps femoris muscle in chronic alcoholic myopathy

***Objective**: to assess the structural and functional state of skeletal muscles in the hip, as well as changes in the content of the sarcomere cytoskeleton proteins titin and nebulin and their phosphorylation level in patients with chronic alcohol intoxication.*

***Patients and methods**. Thirteen patients (4 men and 9 women; mean age, 38.9±9.9 years) with chronic alcohol intoxication were examined. The average duration of regular consumption of alcoholic beverages was 7.6±3.7 years. The mean amount of alcohol consumed per week was 48.2±13.1 units. A control group included 10 healthy volunteers matched for age and sex. A neurological examination was performed according to the generally accepted scheme. Laboratory tests involved blood biochemical analysis estimating the levels of liver enzymes, creatine phosphokinase (CPK), and insulin-like growth factor-1 (IGF-1). All the patients underwent hip magnetic resonance imaging (MRI), followed by assessment of the degree of muscle tissue damage and by determination of the volume of anterior and posterior thigh muscles. The content of titin and nebulin and their phosphorylation level were determined in the muscle tissue samples obtained by an open biopsy from the lateral head of the quadriceps femoris muscle.*

***Results and discussion**. Four (30.8%) patients were found to have proximal leg muscle weakness; the degree of paresis was the same in the anterior and posterior thigh muscles. There was a significant increase in the plasma level of liver enzymes; the CPK level remained within the reference values; there was a tendency towards lower IGF-1 levels. Analysis of MRI data showed that 7 (53.8%) patients had fatty degeneration in the thigh muscles. Quantitative evaluation ascertained a significant symmetrical decrease in the volume of anterior thigh muscles and a tendency towards a symmetrical reduction in that in the posterior thigh muscle compared to the control. Analysis of the content of titin and nebulin in the lateral head of the quadriceps femoris muscle revealed a significant decrease in the percentage of nebulin (81.1%; $p < 0.01$) and intact titin-1 (T1) isoforms (83.6%; $p < 0.01$). The percentage of proteolytic titin-2 (T2) fragments in the muscle of patients did not differ significantly from that in the control group. Estimating the phosphorylation level of the structural muscle proteins showed no significant differences when compared to the control.*

***Conclusion**. Anterior and posterior thigh muscle weakness should be considered as the main clinical manifestation of chronic alcoholic myopathy (CAM) in the absence of biochemical and neurophysiological markers of the disease. Lower extremity muscle MRI that can reveal a lower muscle volume concurrent with fatty degeneration is a non-invasive informative diagnostic technique for CAM. The pathogenesis of skeletal muscle atrophy in chronic alcohol intoxication involves the sarcomere structural proteins titin and nebulin, which regulate the interaction of the major contractile proteins actin and myosin, and whole muscle contraction.*

***Keywords**: chronic alcoholic myopathy; insulin-like growth factor-1; magnetic resonance imaging of hip skeletal muscles; titin isoforms; nebulin.*

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Neuromuscular disorders, which include myopathy and polyneuropathy, are considered as the most frequent effects of alcohol abuse and occur in 50–70% of patients presenting chronic alcohol intoxication. Chronic alcoholic myopathy (CAM), the morphological basis of which is atrophy of muscle fibers, may, along with alcoholic neuropathy, be the cause of patient disability. The pathogenesis of CAM is still not entirely understood. In previous studies, it has been reported that aberrations in the regulatory mechanisms that affect protein synthesis led to the process of muscle atrophy and the development of clinical manifestations of the myopathic syndrome characterized by proximal muscle weakness affecting the lower limbs [1, 2]. There are only a few journal papers available that highlight changes in the major contractile proteins of skeletal muscle after chronic alcohol intoxication. In particular, data on a decrease in the content of giant sarcomeric proteins, titin and nebulin, relative to the content of myosin heavy chains in the hindlimb muscles of chronically alcoholized rats have been obtained [3, 4]. A role of titin

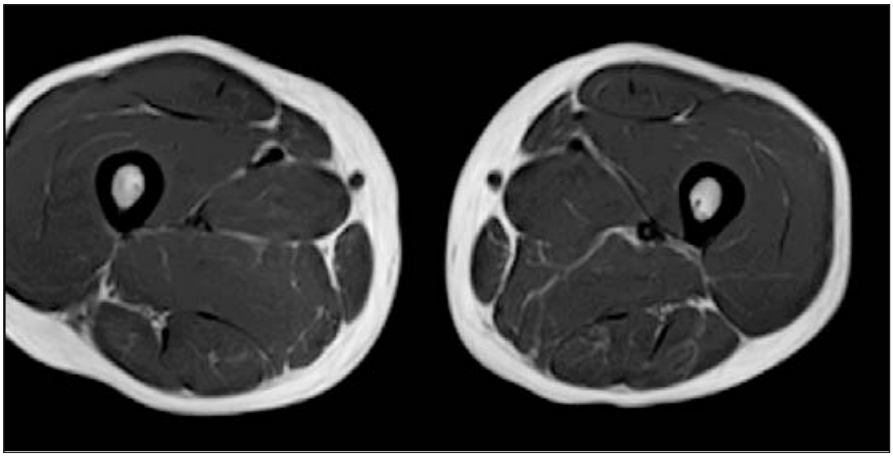

Figure 1. *MRI images of the middle third of the thigh muscles in the volunteer in the control group. _1-weighted image. Axial MRI section*

and nebulin in regulation of interaction between the major contractile proteins actin and myosin in skeletal muscle and for muscle contraction on the whole is discussed.

The objective of the study was to evaluate the structural and functional state of the skeletal muscles of the thigh as well as changes in the contents and phosphorylation levels of the sarcomeric cytoskeletal proteins titin and nebulin in patients with chronic alcohol intoxication.

Patients and methods

A comprehensive clinical and laboratory examination, magnetic resonance imaging (MRI) of skeletal muscles of the thigh in patients with chronic alcohol intoxication, as well as electrophoretic study of changes in the isoform composition and the level of phosphorylation of sarcomeric cytoskeletal proteins titin and nebulin in the quadriceps muscle of the thigh were carried out.

The study involved 13 patients with chronic alcohol intoxication (4 men and 9 women, the average age was 38.9 ± 9.9 years). The average duration of regular consumption of alcoholic beverages in a group of patients was 7.6 ± 3.7 years. The average amount of alcohol consumed per week was 48.2 ± 13.1 units of alcohol, which, according to World Health Organization (WHO) criteria, corresponds to a high risk of developing different effects of alcohol use disorders.

The control group consisted of 10 healthy volunteers (4 men and 6 women who were matched for age (the mean age was 34.4 ± 9.3 years). All subjects in the control group did not drink to excess, had no acute and/or chronic diseases, clinical signs of peripheral nerves and skeletal muscles damage on the moment of examination.

The neurological examination of patients was performed in accordance with the general practice. In the study of motor function, a muscle tone, the strength of proximal and distal muscles of the limbs were evaluated on a six-point scale. In order to identify subclinical reduction of muscle strength in the legs, functional tests were performed: walking on toes and heels, squats.

Laboratory tests included: complete blood count, a biochemical blood test to check levels of liver enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), creatine phosphokinase (CPK) and estimate the IGF-I level (somatomedin C). All patients had MRI of the thigh muscles. Imaging results obtained from a group of patients with chronic alcohol intoxication were compared to those from a control group of patients (Figure 1). Magnetic resonance imaging of skeletal muscles was performed using a 1.5-Tesla Toshiba Excelart Vantage magnetic resonance scanner. The protocol of the study involved scanning with conventional T1SE sequence, repetition time (TR), 500 milliseconds and with T2-weighted- short tau inversion recovery (STIR) sequences as it suppresses the signal from fat, repetition time (TR),4000 milliseconds, of the axial slices through both lower extremity muscles (thigh muscles, 14 mm slice thickness at 1 mm spacing). A scale developed by Mercuri et al [5] was used to analyze imaging findings and evaluate the degree of muscle damage. With this scale it is possible to provide a qualitative evaluation of the degree of fat infiltration in each muscle in the region of interest (Table №1). The identified degenerative changes were assessed taking into account normal age-dependent fatty infiltration within muscles [6].

Quantitative assessment of the volume of the anterior and posterior muscles of the thigh was carried out in patients with CAM and in individuals in the control group. To measure the volume of the skeletal muscle of the anterior and posterior muscles of the thigh, the lateral head of the quadriceps muscle and the biceps of the thigh were used, respectively.

The volume of skeletal muscle was calculated with Simpson's rule (stereometric formula of the median section) [7].

$$V=(d/3)*[(A1+A7) +4(A2+A4+A6)+2(A3+A5)],$$

where V was the volume of the muscle of interest;

An was muscle cross-sectional area;

d was the distance within which the slice is limited by two parallel planes (Fig. 2).

The calculation of muscle areas was made by segmentation of each muscle on each MR-section using Onis 2.5, a free MRI viewing software.

Determination of the titin and nebulin content. Muscle tissue samples were obtained by means of open biopsy from the lateral head of the quadriceps femoris muscle of the thigh using Novocaine 1% solution as a local anesthetic. Samples were stored at – 75°C. Separation of nebulin (molecular mass of 890 kDa), isoforms of intact titin-1 (highmolecular weight of T1, 3500–3700 kDa) and proteolytic fragments of titin (T2- frag-

Table 1. A scoring system (modified according to [5]) to rank muscle tissue atrophy after analysis performed on T1-weighted images.

Score	Grades of muscle tissue atrophy
0	Normal muscle tissue
1	Early "moth eaten" appearance in muscle fibers with small areas of increased MR signal intensity
2a	Late "moth eaten" appearance in muscle fibers with numerous discrete areas of increased MR signal intensity with beginning confluence, comprising less than 30% of the volume of the individual muscle
2b	Late "moth-eaten" appearance, with numerous discrete areas of increased MR signal intensity with beginning confluence, comprising 30–60% of the volume of the individual muscle
3	Washed-out appearance, fuzzy appearance due to confluence of not less than 3 areas in one muscle with an increase in MR signal intensity
4	End-stage appearance, muscle replaced by increased density connective tissue and fat with only rim of fascia and neurovascular structures distinguishable

ments, 2000–2200 kDa) was performed in the presence of sodium dodecyl sulfate (SDS) by electrophoresis through large-pore 2.2% polyacrylamide gel and agarose with concentration of 0.5–0.6% [8]. The gels stained with Coomassie Brilliant Blue (G-250 and R-250, mixed in a 1:1 ratio) were digitized, and Total Lab v1.11 was used to compare the density of bands on digital gel images. The content of titin and nebulin was evaluated relative to the content of myosin heavy chain (MHC) (Figure 3).

Determination of the level of titin and nebulin phosphorylation. This analysis was carried out to elucidate whether this post-translational modification has an effect on the change in sensitivity of the above-mentioned proteins to proteolysis. The native level of protein phosphorylation in the gel was assessed by Pro-Q Diamond fluorescent dye (Invitrogen) for staining phosphoproteins. The gels were stained with the above-mentioned fluorescent dye for 1.5 hours, and subsequently washed in Pro-Q Diamond destain solution. Phosphate-containing protein bands were visualized with the Bio-Rad Chemi Doc Touch Imaging System. Gels were then stained with Coomassie Brilliant Blue G-250 and R-250, mixed in a 1:1 ratio, to compare the protein content to that in the control sample (Figure 3).

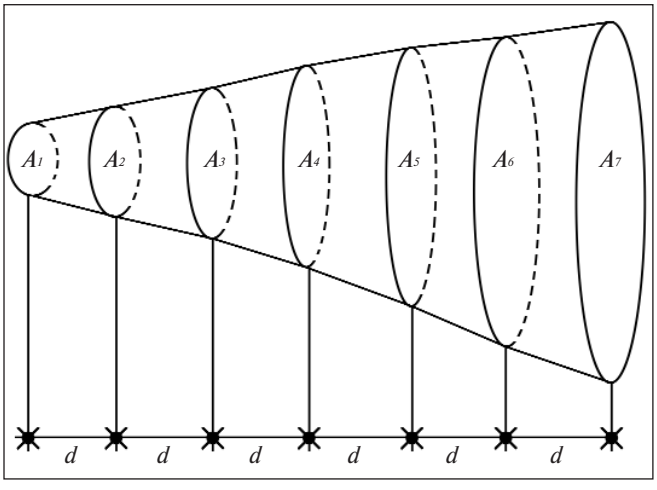

Figure 2. *Calculation of the volume of skeletal muscle with Simpson's rule*

Statistical processing of the results. For the evaluation of laboratory and MRI data, one-factor dispersion analysis was used, the main idea of which was to apply the Fisher criterion to estimate the difference between the average intergroup and average intragroup dispersions. Statistical processing of electrophoretic data was carried out using the nonparametric Mann–Whitney U-test. Data on the change in the protein content and in the phosphorylation level of T1 and T2 were summarized in Tables, control values were taken as 100% [9].

To obtain descriptive statistics, the scientific professional statistical package Statistica 10.0 from StatSoft was used.

Results. Complaints of weakness in legs and difficulty in walking, associated with it, presented 5 (38.5%) patients. Two of them (15.4%) experienced difficulties when standing up from a squatting position. 12 patients (92.3%) reported sensory disorder in the legs, which ranged from mild numbness or tingling to intense shooting pains. Four (30.8%) patients complained of burning sensation in feet; 6 (46.2%) patients noted stagger when walking and disorder of the feeling the floor under their feet.

Clinical neurological examination revealed the presence of proximal weakness in the leg muscle in 4 (30.8%) patients: in 3 patients weakness was rated 4 and the sign of it was trouble with climbing stairs and inability to walk out of a deep squat position, and in 1 patient muscle weakness was rated 3, movement possible but not against resistance by the patient and the use of additional means of support was required. In 3 (23.1%) patients, distal paraparesis of lower limbs was identified during examination, muscle strength was rated 3. In all cases, the anterior and posterior muscles of the thigh and lower leg had similar degrees of paresis.

In the study of tendon reflexes, 7 (53.8%) patients showed a similar decrease or loss of Achilles reflexes. Knee reflexes were absent in 3 (23.1%) patients, and in another 3 (23.1%) patients they were similarly diminished.

Sensory disturbances in the lower lilmb muscles were detected in 11 (84.6%) patients and represented decreased sensitivity to pain and temperature, hyperalgesia with elements of hyperpathy and tactile allodynia, decreased muscle-joint and vibration sensitivity, of polyneuropathic type. In 9 (69.2%) cases, the atactic syndrome was observed: sensitive ataxia was detected in 2 (15.4%) patients, cerebellar ataxia was observed in 5 (38.5%), mixed ataxia as combination of cerebellar and sensitive ataxia was found in 2 (15.4%) patients.

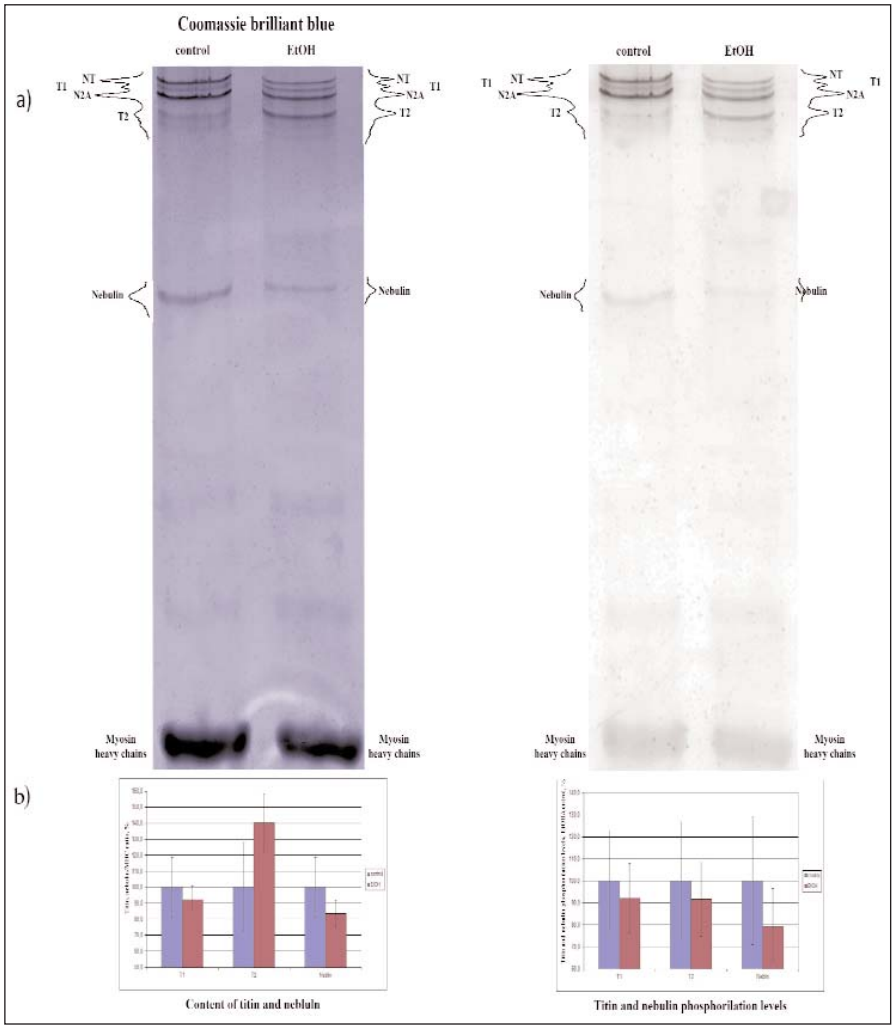

Figure 3. *Changes in titin and nebulin contents and in their phosphorylation levels in the quadriceps femoris muscle (control – alcohol).*
Electrophoregram of proteins (a) and diagrams illustrate changes in the titin and nebulin contents and phosphorylation level as opposed to control (b). The T1 band is titin 1 (NT, N2A), the T2 band is titin proteolytic fragment

Table №2. Data on the study of liver enzymes.

Liver enzymes	Patients with CAM (n=13)	Control (n-10)
AST	60,2±19,7 *	18,5±1,2
ALT	37,4±7,1 *	19,8±2,8
GGT	248,1±116,1*	18,8±1,6
*p<0,05		

According to the laboratory test results, a significant increase in the level of liver enzymes (AST, ALT and GGT) was found in patients with CAM when compared to that in the control group. This may indicate a long-term toxic effect of ethanol and its metabolic products on liver function (Table 2).

The CPK level in blood plasma in all examined patients fell within the reference interval, the average value was 67.6 ± 10.6 u/l, which was not significantly different from this value in the control group (62,2 ± 3.28 u/l) . The study of the content of insulin-like growth factor-I (IGF-I or somatomedin C) showed that there is a tendency of its decrease in the group of patients with CAM. This may indicate the impaired hepatic plasma protein synthesis. The average level of IGF-I was 169.2 ± 26.3 ng/ml in the group of patients and 218.3 ± 15.3 ng/ml in the control group.

MRI of the thigh muscles in the examined patients showed no signs of swelling of the muscle tissue, which made it possible to exclude the inflammatory nature of myopathy. Analysis of MRI data showed the presence of fatty degeneration in the thigh muscles in 7 (53.8%) patients: in 1 case changes were observed only in the anterior muscles, in 3 cases fatty degeneration was seen only in the posterior muscles, and another 3 patients demonstrated the presence of fatty degeneration in the anterior and posterior thigh muscles (Figure 4). The degree of degeneration using the scale proposed by E. Mercuri [5] ranged from 1 to 2 points (Table 1).

Quantitative estimation of the volume of thigh muscles revealed a significant symmetric decrease in the volume of the anterior muscles (lateral head of the quadriceps muscle of the thigh) in patients with CAM as compared to that in the control group and a tendency to a symmetric decrease in the volume of the posterior muscles (biceps of the thigh) (Table 3).

Analysis of the content of titin and nebulin in the lateral head of the quadriceps muscle of the thigh showed a significant decrease in the percentage of nebulin (81.1%, p <0.01) and isoforms of intact titin T1 (83.6%, p <0.01). The percentage of proteolytic T2-fragments (degradation products) of titin (100.3%, p> 0.05) in the muscle of the patients did not differ significantly from the control group.

Assessment of the level of phosphorylation of structural muscle proteins showed no significant differences in comparison with the control: the level of phosphorylation of intact T1 isoforms was 97.1%, the degree of degradation products T2 and nebulin was, 115.3% and 97.8%, respectively.

Discussion

Neurological examination of patients with chronic alcohol intoxication revealed neuromuscular manifestations of alcohol disease. The most frequent complication was alcoholic polyneuropathy (APN), which was observed in 11 (84.6%) patients. This number is slightly higher than that reported on the frequency of APN and may be associated with a larger group of inpatients [10].The polyneuropathy type sensory disturbances varied from

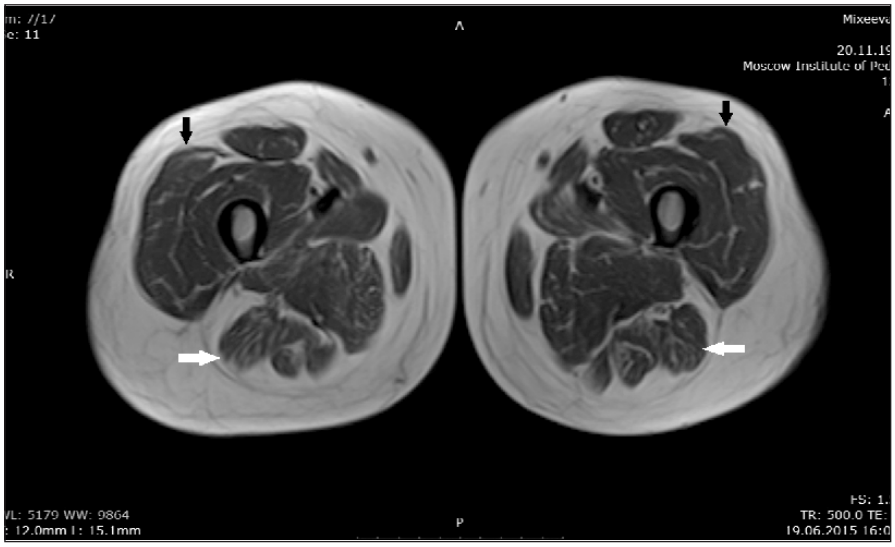

Figure 4. *MRI images of the middle third of the thigh muscles in the patient with signs of fatty degeneration. T1-weighted image. Axial MRI section. Black arrows point out changes in the anterior muscles (lateral head of the quadriceps muscle of the thigh – 1 point according to Mercuri), white arrows point out changes in the posterior muscles of the thigh (biceps muscle of the thigh – 2a points according to Mercuri)*

Table 3. Data on the thigh skeletal muscle volume.

Muscle	Patients with CAM		Control	
lateral head of the quadriceps muscle of				
the thigh, cm^3				
on the right	336,3±54,6		491,8±107,8	
on the left	325,5±63,5**		485,1±104,7	
biceps of the thigh, cm^3				
on the right	180,3±96,1		261,1±103,1	
on the left	171,4±97,8		255,4±101,8	
*p<0,01				

mild impairment of skin sensitivity and paresthesias to a drastic decline in cutaneous and subcutaneous sensation in feet. In 3 (23.1%) patients, movement disorders characteristic of distal symmetric polyneuropathy were also detected, in the form of weakness of the feet, mainly in the extensor muscle group. In 7 (53.8%) cases, there was a decrease or loss of Achilles reflexes.

Clinical manifestations of the myopathic syndrome in the form of slowly progressive muscle weakness in the proximal areas of legs were detected in 4 (30.8%) patients. According to the literature, CAM occurs in 40–60% of cases of chronic alcohol intoxication and is characterized by the absence of the typical myopathic pattern according to the needle electromyography data and normal CPK level [11–13]. In this study the CPK level was not higher than the reference range, as evidenced by the absence of muscle tissue necrosis. It has been shown that the content of IGF I tended to be lower in blood serum, as would be the case for a disorder of the systemic mechanisms of protein synthesis in patients with chronic alcohol intoxication [14].

A thigh muscle biopsy recognized as the "gold standard" for diagnostics of CAM was followed by a morphometric and immunohistochemical study undertaken to help identify signs of muscle fiber atrophy, mainly type 2 (fast) [11, 12, 15]. However, the morphological method is invasive and subsequent examination of muscle biopsy samples is complex, that is why their widespread use in clinical practice is limited. Taking into account that MRI of skeletal muscle provides valuable information allowing a non-invasive investigation of muscle function conducted for the diagnosis of neuromuscular diseases, MRI of the thigh muscles of

alcohol-abusing patients has been performed to diagnose CAM. An MRI examination revealed the presence of fatty degeneration in the thigh muscles in 7 (53.8%) patients. Changes more often affected the posterior muscles, experiencing the maximum load in the vertical position. In most cases, the degree of fatty degeneration was moderate (stage 1 to stage 2a according to the scoring system described by E. Mercury), and only one case reached stage 2b, ie, it affected from 30% to 60% of the muscle studied. The atrophic changes were distributed symmetrically and prevailed in the anterior compartment of thigh muscles. Degenerative changes of the studied muscles, as well as their atrophy, were rather frequently detected in MRI images than during clinical neurological examination. Consequently, MRI of the thigh muscles makes it possible to detect the early preclinical stage of CAM.

Experimental data obtained have shown that there are changes in the content and phosphorylation of structural muscle proteins such as titin and nebulin in the presence of chronic alcoholization. Titin and nebulin are giant proteins of the sarcomeric cytoskeleton of the striated muscles of vertebrates [16]. Titin was discovered by two independent groups of researchers, using gel electrophoresis. Two unknown protein bands with a molecular mass of more than 1000 kDa were seen in the electrophoregrams of the skeletal muscles of the rabbit and chicken. The proteins appeared to be immunologically identical and named titin-1 (T1) and titin-2 (T2). The latter is a proteolytic fragment of titin-1 [17]. In sarcomers of the heart and skeletal muscles, titin is the third protein in terms of number (after actin and myosin). It has been shown that titin serves as a template for the assembly of the thick myosin filaments of the sarcomere [18]; is involved in maintenance of muscle contractile function [19]; and in regulation of actin-myosin interaction. The elastic protein titin plays an important role in regulation of protein metabolism in the sarcomere [20]. Nebulin is a giant protein that stabilizes actin filaments and participates in regulation of the actin-myosin interaction [16, 21]. The results of experimental studies suggest that protein metabolism in muscle cells is initiated by proteolysis of titin, nebulin and other sarcomere proteins, followed by degradation of their fragments to amino acid residues [22]. The decrease in the content of titin and nebulin due to their increased proteolysis, observed during the progression of muscle atrophy [17], leads to disruption of a highly ordered sarcomere structure, deterioration of elastic properties and contractility of muscles.

We revealed a decrease in the content of titin (T1) and nebulin in the quadriceps femoris muscle of patients with chronic alcohol intoxication; it was probably due to the activation of proteolysis processes. Increased proteolytic degradation of titin and nebulin may be a result of change in the level of phosphorylation of these proteins and an increase in their sensitivity to proteolysis. This assumption is not without foundation. We have not found in the scientific literature direct experimental evidence of an increased sensitivity of titin or nebulin to proteolysis due to changes in its level of phosphorylation,. However, there are indirect data which indicate that an increase in the degree of phosphorylation of titin is accompanied by an increased proteolytic degradation of this protein. Similar changes in the level of T1 and T2 phosphorylation, occured with increased proteolysis of intact titin, were observed during the progression of alcohol-induced atrophy in the gastrocnemius and soleus muscles of rats [3]. Also, there is some evidence that the increased level of phosphorylation of titin in the quadriceps muscle of the thigh in patients with Ehlers–Danlos syndrome was accompanied by a decreased content of this protein [23]. It has been suggested that hyperphosphorylation of titin, mainly the T2-part of its molecule, contributes to an increase in the sensitivity of this protein to proteolysis [3].

Taking into account this assumption and the results showing that titin and nebulin content declined in the quadriceps muscle of the thigh in the examined patients with CAM, hyperphosphorylation of titin and nebulin was expected to be observed. The results obtained did not confirm the assumption. Despite the tendency to a decrease in the phosphorylation level of T1 and nebulin, no significant differences were evident in phosphorylation in the muscles of the patients.

We attempt to explain this phenomenon. It is known that the disbalance between protein synthesis and decay, which is induced by chronic alcohol intoxication, underlies the development of CAM [24, 25]. Recent studies showed that in the early phases of alcohol abuse, the patients demonstrate changes in regulation of anabolic and catabolic signaling pathways that precede the development of skeletal muscle atrophy and the appearance of clinical symptoms of alcoholic myopathy [25]. Most probably, in this case titin (and may be nebulin) hyperphosphorylation occurs leading to an increased proteolysis of this protein and a decrease in its content, that, in turn, may contribute to the development of atrophic changes in the muscles of the patients. In the late phases of alcohol abuse, the process-controlled molecular orientation affecting the phosphorylation level of titin and nebulin, apparently changes resulting in hyperphosphorylation of these proteins. A decrease in phosphorylation may enhance titin and nebulin susceptibility to proteolytic cleavage and maintain even reduced but relatively stable content of these proteins in the sarcomere. In favor of this assumption there is evidence of the lack of an increase in the content of proteolytic T2-fragments in the quadriceps muscle of the thigh in patients. This may be a result of the slowed proteolytic cleavage of titin.

Conclusion

In conclusion, CAM is the most common but rarely diagnosed alcoholic disorder. The wasting of the thigh muscle, with more pronounced muscle weakness in the antigravity posterior muscles is considered to be the main clinical manifestation of CAM in the absence of biochemical and neurophysiological markers for the disease. MRI of the lower extremeties, that may show a decreased volume of the muscle and fatty infiltration, is an informative technology enabling noninvasive diagnosis of CAM. The structural sarcomeric proteins titin and nebulin which are involved in regulation of interaction between actin and myosin, the main contractile proteins, and function in muscle contraction in general, contribute to the pathogenesis of the atrophic process of skeletal muscles in chronic alcoholic intoxication.

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